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Under standard laboratory conditions of rectangular light dark cycles and constant warm temperature, *Drosophila melanogaster* show bursts of morning (M) and evening (E) locomotor activity and a 'siesta' in the middle of the day. These M and E components have been critical for developing the neuronal dual oscillator model in which clock gene expression in key cells generates the circadian phenotype. However under natural European summer conditions of cycling temperature and light intensity an additional prominent afternoon (A) component that replaces the siesta is observed. This novel A component has been described as an 'artifact' of the TriKinetics locomotor monitoring system that is used by many circadian laboratories world-wide. Using video recordings we show that the A component is not an artifact, neither in the glass tubes used in TriKinetics monitors nor in open field arenas. By studying various mutants in the visual and peripheral and internal thermo-sensitive pathways, we reveal that the M component is predominantly dependent on visual input whereas the A component requires the internal thermo-sensitive channel TrpA1. Knockdown of TrpA1 in different neuronal groups reveals that the reported expression of TrpA1 in clock neurons is unlikely to be involved in generating the summer locomotor profile, suggesting that the internal AC TrpA1 neurons are responsible for the A component. Studies of circadian rhythms under semi-natural conditions therefore provide additional insights into the molecular basis of circadian entrainment that would otherwise be lost under the usual standard laboratory protocols.

circadian | Drosophila | behavior | locomotor | afternoon

Introduction

The circadian clock infiltrates almost every aspect of behavior and physiology of higher organisms and even some bacteria. Most studies of 24 h rhythms are carried out under strictly controlled laboratory conditions, an approach leading to a remarkably informative dissection of the clock, whose main molecular cogs are conserved among vertebrates and insects. Laboratory experiments are often extrapolated to the wild with the assumption that they reflect the natural situation. However, recent semi-natural studies in mice, hamsters, and *Drosophila,* have revealed a some unexpected findings. For example, the widely-held belief from laboratory studies that mice and golden hamsters are nocturnal needs to be revised because in the wild they are predominantly or exclusively diurnal (1, 2). Similarly in *D. melanogaster,* locomotor rhythms studied in semi-natural conditions reveal that deeply held, laboratory-derived assumptions, may require significant revision. These include the crepuscular nature of fly activity, the role of the clock in 'morning anticipation' and midday 'siesta', the requirement for clock gene expression in the central clock neurons for entrainment, and the role of light-dark cycles as the most important environmental Zeitgeber ('time-giver') in entraining the clock (3).

Vanin et al (3) observed that in the wild the phase of various features of circadian locomotor behavior such as the Morning (M) and Evening (E) components were best predicted by temperature, rather than 'anticipation' of dawn and dusk over the seasonal light-dark cycle. In addition, at the warmer temperatures of European summers, flies did not generate an afternoon 'siesta' as in the laboratory. Instead, they dramatically increased their activity so that the major component of their locomotor profile was now the newly described Afternoon (A) peak. The phase of the A component was modulated by mutation at the *period (per)* locus suggesting that A represented a clock-mediated escape response from heat induced stress (3, 4). Most surprisingly, null mutants of the negative regulators of the circadian clock *period (per^{01})* and *timeless (tim^{01})* exhibited naturally entrained behavioral profiles largely indistinguishable from those of wild-type strains. In sharp contrast, under laboratory conditions of constant 25°C temperature and rectangular LD cycles, per^{01} and tim^{01} flies show no anticipation of dawn/dusk and these mutants simply react to light-on or light-off signals with startle effects (5). The anticipatory nature of the M and E components in the laboratory led directly to the development of the dual oscillator model in the fly in which the Pigment Dispersing Factor (PDF) expressing s-LNv and l-LNv cells generate the M locomotor component,

Significance

The study of laboratory generated circadian locomotor activity patterns of *Drosophila*, played a critical role in determining how fruitfly (and mammalian) clocks function. However recent observations of fly activity in the wild challenged many assumptions about how the clock might work. A new prominent summer locomotor component emerged called 'A' (afternoon), which replaced the laboratory 'siesta'. The A component has been criticised by others to be an artifact, but our study here shows that it is genuine and is observed under a variety of simulated natural conditions. The A component is temperature and clock-dependent and is generated by expression of the internal thermosensor TrpA1, revealing a novel pathway for environmental input to the clock.

Reserved for Publication Footnotes

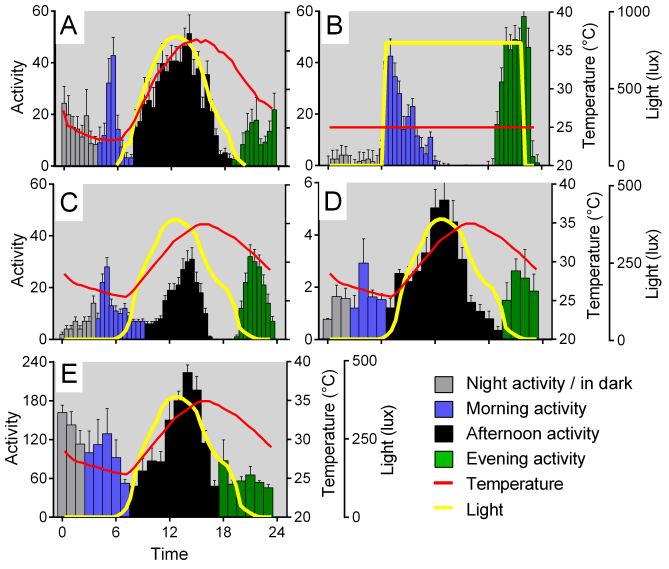

Fig. 1. - Natural simulations in the laboratory support the existence of an A component.A: Flies recorded in the wild (n=9 male HU recorded 28/07/2007, day mean temperature = 29.7°C, max light 842 lux) clearly reveal the additional and major afternoon (A, black) peak of activity (mean activity ±sem). **B**: Standard laboratory conditions at constant 30°C, LD16:8 (32 male HU, LD 18:6 700 lux) reveal no A peak. **C-E**: Step-free semi-natural simulation reveals M, A and E peaks of activity, whether measured in tubes using TriKinetics monitors (**C**, n=32) or virtual beam crossing analysis (**D**, n=9, plotted as mean ±sem of 3 replicates), or measured in open field arenas (**E**, total distance travelled by 4 male and 4 female flies, averaged across 3 replicates).

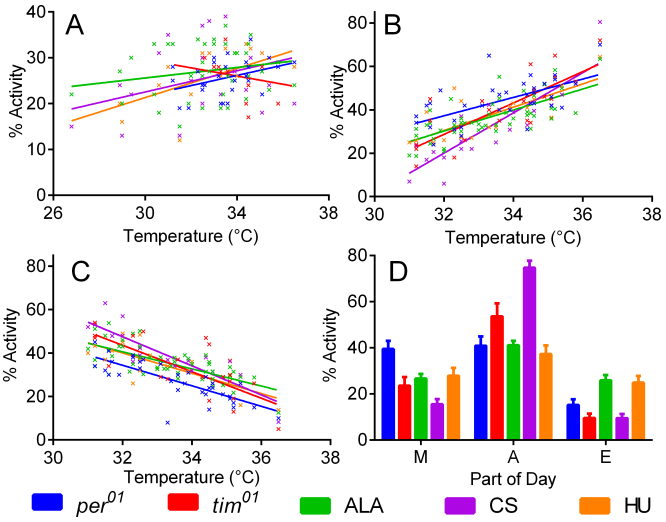

Fig. 2. - Relationship between temperature and M A and E locomotor components under semi-natural conditions.A: Mean (±sem) daily % of M activity against maximum temperature of previous day; R^2 values are not significant (per^{01} n=26, $R^{2=}$0.143, P=0.057; tim^{01} n=17, $R^{2=}$0.094, P=0.231; ALA n=34, $R^{2=}$0.053, P=0.19; CS n=16, $R^{2=}$0.151, P=0.137), except for HU (n=16, $R^{2=}$0.473, P=0.003). **B**: Mean daily % A activity against corresponding day's maximum temperature. All R^2 values are significant (per^{01} n=36, $R^{2=}$0.397, P<0.001; tim^{01} n=22, $R^{2=}$0.624, P<0.001; ALA n=49, $R^{2=}$0.508, P<0.001; CS n=20, $R^{2=}$0.728, P<0.001; HU n=20, $R^{2=}$0.54, P<0.001). **C**: Same as B but for mean daily % E activity. All R^2 values are significant (per^{01} n=36, $R^{2=}$0.623, P<0.001; tim^{01} n=22, $R^{2=}$0.582, P<0.001; ALA n=49, $R^{2=}$0.495, P<0.001; CS n=20, $R^{2=}$0.741, P<0.001; HU n=20, $R^{2=}$0.566, P<0.001). **D**: Locomotor activity under laboratory simulations of warm summer day (25-35°C cycles, maximum light 500 lux). tim^{01} shows significantly higher A and lower E than congenic HU. Canton-S shows significantly different M, A and E than the ALA and HU wild-types (see text for full description of results).

whereas the more dorsal clock neurons (LNds and DNs) produce the E component (6, 7).

While Vanin et al focused predominantly on the phases of the major locomotor components under natural lighting and thermal conditions (3), in a similar natural study, Menegazzi et al suggested that although *per* null mutants look similar in their behavioral phasing to wild-type, the A peak tends to be larger in per^{01} mutants (4). These authors suggested that PER normally serves to reduce the amount of 'inappropriate' activity that occurs during the warmest part of the day (4). While their results were based on a very small sample of flies on a few days of recordings, they were nevertheless welcomed in that they revealed that possessing a wild-type clock appeared to be behaviorally adaptive compared to having a severely disturbed clock.

Another study performed under semi-natural conditions at tropical latitudes has questioned the validity of the A component (8). These authors suggest instead that A represents a behavioral artifact as a result of flies avoiding the midday sun by sheltering in the shaded part of the glass activity tube where the TriKinetics infra-red detectors are located, leading to inappropriate triggering of the sensor and high activity counts. In apparent support of this model they observed that flies in open field Petri dish arenas did not show an A component under summer conditions, though this interpretation has been criticised (9), in part because Petri dishes are well known to be problematic for Drosophila open field behavioral recordings (10).

Given the interest generated by Vanin et al (3), we have revisited these natural studies and extended them with more sophisticated simulations of natural temperature and light cycles in the laboratory. By using video recordings of fly circadian activity in glass tubes and open field arenas we investigate whether the A component is an artifact. Furthermore, in both the Vanin et al (3) and the Menegazzi et al (4) studies, the classic *per* mutants were congenic with each other but were compared to three different wild-type strains so genetic background was not controlled. Using congenic controls we re-examine whether we can observe a phenotype for arrhythmic mutants in simulated semi-natural conditions. Finally we study the A peak in a range of photoreceptor and thermoreceptor mutants in order to investigate the underlying genetic and neuroanatomical basis for this novel summer element of circadian behavior.

Results

The A component is not an artifact

Fig 1A shows the locomotor profile of HU wild-type flies using TriKinetics monitors recorded in the wild on an Italian summer's day with naturally varying temperature and light cycles (max 840 lux, mean temperature 29.7°C). Fig 1B illustrates the results from HU flies in the standard laboratory paradigm at a constant temperature of 30°C in rectangular 700 lux light-dark cycles (LD16:8). The main difference between the two figures is the presence of the A (afternoon) component. By simulating a warm Italian midsummer day using smooth changes in temperature (25-35°C, Fig S1A) and light intensity (max 500 lux, Fig S1B) we were able to induce an activity profile with clear M, A and E components (Fig 1C) very similar to that observed in the wild (Fig S1C-D). As in the wild, the A peak is not prominently expressed with a 20-30°C thermal cycle (Fig S1E-F).

De et al (2013) suggested that the A component is an artifact because on warm sunny days the flies seek the shaded area between the emitter and detector in the TriKinetics DAM2 recording system, thereby over-activating the infrared beam which generates the activity counts. Although Vanin et al recorded their data in completely shaded conditions we addressed this issue by mounting the glass activity tubes from TriKinetics onto an unshaded white background, and recorded infrared video of their

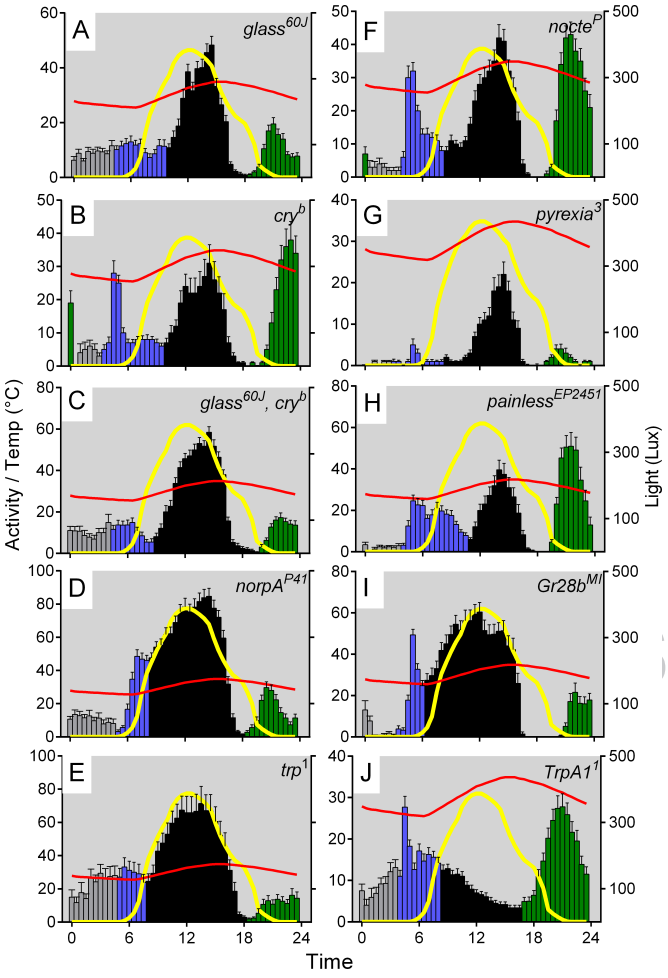

Fig. 3. – The effects of mutations in photo- and thermos-receptor pathways on laboratory semi-natural locomotor profiles Mean locomotor activity (±sem). Color key as in Fig 1. **A**: *glass*^{60J} n=28, **B**: *cry*^{b} n=17, **C**: *glass*^{60J}, *cry*^{b} n=27, **D**: *norpA*^{P41} n= 31, **E**: *trp*^{1} n=20, **F**: *nocte*^{P} n=29, **G**: *pyx*^{3} n=22, **H** *pain*^{EP2451} n=24, **I**: *Grb28b*^{MI} n=23, **J**: *TrpA1*^{1} n=30.

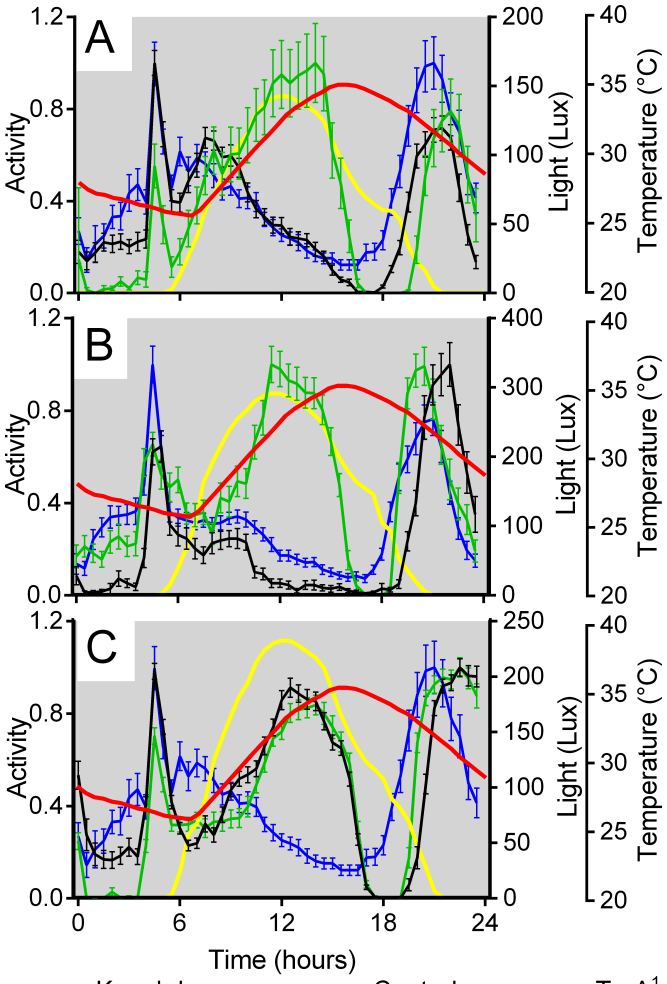

Fig. 4. - The effects of knockdown or overexpression of TrpA1 on the A peak of locomotor behavior.A: Knockdown of *TrpA1* using *TrpA1*^{Gal4} is sufficient to recapitulate lack of evening peak. Knockdown: *TrpA1*^{GAL4} */TrpA1-IR*^{JF02461} (n=30), Control: *TrpA1*^{GAL4} (n=20), mutant: *TrpA1*^{1} (n=30). **B** TrpA1 is required in neurons to give afternoon peak. Knockdown: *elavGal4; UAS>Dcr2; TrpA1-IR*^{JF02461} (n=36), Control: + / *TrpA1-IR*^{JF02461}(n=30), mutant: *elavGal4; +; TrpA1*^{1} (n=26). **C**: *timGal4* knockdown of *UAS-TrpA1* has no effect on the A component. Knockdown: *UASDicer2, timGal4; UASTrpA1-IR*^{JF02461}(n=32), Control: *UASDicer2, timGal4; mCherry* (n=29), mutant: *TrpA1*^{1} (n=30). Data scaled to maximum daily peak, mean ±sem.

activity under semi-natural conditions (depicted in Fig S2). Using ActualTrack™ software we simulated a 'virtual' light beam across the centre of each tube, and counted the number of times flies crossed this beam. Our results show that monitoring the flies in this manner results in activity records with clear M, A and E components (Fig 1D), contradicting the suggestion that the A component is an artifact of shade within the TriKinetics DAM2 system.

De et al further claimed that observations of flies' open field behavior in Petri dishes showed an absence of the A component, implying that the A component might only be observed under the restricted spatial environment of the glass tubes (and shade) inherent in the TriKinetics system. We recorded the activity of groups of four male and four female flies in open field chambers developed by the Dickinson laboratory (11), and used ActualTrack™ software to determine the total movement of flies recorded under infrared light for 5 of every 30 min under unshaded semi-natural conditions. Again the results clearly show the A component as the major part of the locomotor activity profile under simulated warm summer conditions, with M and E components providing smaller contributions (Fig 1E). Consequently, the A component is observed in TriKinetics monitors, in isolated glass tubes and in open field chambers (Fig 1A,C-E); indeed De et al's incorrect conclusion was based on a misinterpretation of their own data (see Discussion).

Do arrhythmic mutants show any locomotor phenotypes in semi-natural conditions?

Menegazzi et al suggested that the amplitude of the A component could be modulated by clock mutations (4). We re-interrogated the extensive Vanin et al database (3) by dividing the day into those segments that represented M activity (02:30 to 08:00), A activity (08:00 to 16:30), and E (16:30 to 22:00) with night activity (N) falling between 22:00 and 02:30. Data were expressed as a % total daily activity falling within these segments and all data were taken from Italian summer recordings between Jun 19-Sept 3. We selected days in which the maximum temperature exceeded 31° C expecting to observe a strong A response and correlated each locomotor component with maximum daily temperature (Fig 2).

Fig 2A shows the relationship of % M activity with the maximum temperature of the previous day. From the R^2 values a very weak and non-significant relationship for both *per*^{01} and

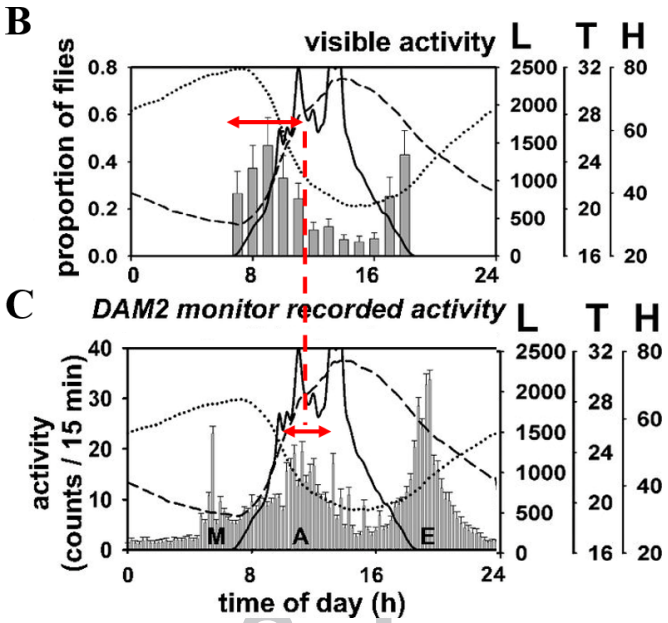

Fig. 5. - The A component is not an artifact even in De et al's results. The TriKinetics and corresponding visual tracking results reported by De et al (8) from their Fig 2. By placing one panel over the other it is clear that the DAM2 TriKinetics M component labelled by De et al occurs at ~05.00 am, several hours before the visually monitored peak that they incorrectly refer to as 'M' and which occurs between 08.00-11.00 am. The visually monitored 'M' overlaps substantially with the A component observed in TriKinetics (red dotted line).

tim^{01} mutants and two of the three wild-types is observed. Only the HU wild type (from Holland) shows a significant correlation with temperature, but linear regression of the dataset shows no significant difference between genotypes either in slope ($P = 0.105$) or intercept ($P = 0.334$). The A component shows a much stronger positive relationship with the corresponding day's maximum temperature for all genotypes (see R^2 values) and there are significant differences between genotypes in the slope ($P = 0.007$) with *per*^{01} having slightly more A component than the other genotypes over the complete temperature range, and *tim*^{01} and Canton-S having the highest levels of A at the very highest temperatures (Fig 2B). The amplitude of the E component has a strong negative relationship with temperature (Fig 2C) but with no significant difference in slope among lines ($P = 0.239$). Thus the extensive results from semi-natural conditions provide some support for Menegazzi et al's suggestion that *per*^{01} mutants show elevated levels of A activity.

When we simulated a warm summer's day in the laboratory using the two arrhythmic mutants that are now congenic with HU, and all three wild-type strains, we observe a number of differences (interaction genotype x MAE $F_{8, 264} = 18.44$, $P < 0.0001$, Fig 2D). The two recently isolated wild lines (HU, ALA) show very similar patterns of behavior ($P > 0.9$ for M, A and E), but are significantly different from the laboratory Canton-S strain (HU vs CS, $P(M) = 0.034$, $P(A) < 0.0001$, $P(E) = 0.0035$), revealing a large effect of genetic background. When comparing clock mutants outcrossed into the HU background, *tim*^{01} but not *per*^{01} shows greater A activity than its corresponding wild-type (Tukey *post-hoc* $P=0.0021$) but less E ($P= 0.0046$).

Molecular underpinnings of the A peak

The semi-natural locomotor recordings made by Vanin et al (3) revealed that temperature played a crucial role in the expression not only for the A component, but also for M and E. However, while the M component appeared to be a temperature-dependent response to twilight with little or no clock gene influence, the E component was temperature *and* clock-gene dependent (3). We further investigated the effects of light and temperature in our semi-natural incubator paradigm by examining the behavior of backcrossed mutant strains (to HU) with restricted abilities to sense their environment.

Under simulated warm summer conditions, mutants with a compromised photo-transduction pathway (left hand panels of Fig 3), either as a result of the morphological loss of photoreceptor cells as in *glass*^{60j} or the double mutant *glass*^{60j} *cry*^{b} (Fig 3A,C), a deficient Phospholipase C-β as in *norpA*^{P41} (Fig 3D) or in the cation-specific calcium channel *trp*^{1} (Fig 3E), exhibited a relative reduction in the amplitude of the M and E components compared to HU, with a corresponding increase in A (Fig S3, also includes statistical analysis). *cry*^{b} circadian blue-light photoreceptor mutants in contrast displayed robust M, A and E peaks and under these conditions and were not significantly different from HU (Fig 3B, Fig S3A). We also examined the effect of mutations in genes known to contribute to temperature sensing in the range 25-40°C (right hand panels of Fig 3), including the Trp channels *TrpA1* (12), *painless* (13) and *pyrexia* (14), the temperature entrainment mutant *nocte* (15), and the gustatory receptor paralogue *Gr28b* required for rapid negative thermotaxis (16). The most dramatic differences observed involved *pyrexia*, in which the M and E components were significantly suppressed compared to A, and *TrpA1* mutants in which the A component was largely eliminated (Fig 3F-I, Fig S3).

Reported expression of TrpA1 in clock neurons is not required for the A peak

It has been reported that as well as being expressed in a number of brain regions, TrpA1 is also expressed within some of the cells that make up each sub-cluster within the LNv and DN clock neurons (17). Consequently we knocked down *TrpA1* expression using RNAi using different Gal4 drivers. Knockdown of *TrpA1* using either a *TrpA1*^{Gal4} (Fig 4A) or the pan-neuronal enhancer trap *elavGal4* (Fig 4B) was sufficient to mimic the behavior of *TrpA1*^{1} mutants with a complete lack of A component. However, *TrpA1* knockdown in clock neurons using *timGal4* did not recapitulate the lack of an A peak, even when co-expressing *UAS-Dicer2* to enhance the potency of the RNAi (Fig 4C). Thus it would appear that limiting *TrpA1* knockdown to the clock cells does not reduce the A component.

Discussion

Among several unexpected results of the semi-natural studies of locomotor activity of Vanin et al, the most attention has been generated by the novel finding that flies are highly active under warm natural conditions during the afternoon, giving rise to the A component (3). This observation was at odds with conventional laboratory studies at constant warm temperatures of 25°C or above that reveal that flies take a 'siesta' in the afternoon, a phenotype that has been associated with *per* alternative splicing in a number of studies (18-20). De et al proposed that the A component is an artifact of the flies seeking the shaded part of the TriKinetics monitors in which the infra-red detector is situated. According to them, flies 'fidgeting' while they are stationary within light beam, generates spurious activity counts. We have shown conclusively using analysis of video recordings in both unshaded glass tubes and in open field arenas that the A component forms a major part of the circadian activity profile under summer conditions, fully consistent with the TriKinetics semi-natural recording of Vanin et al (3). Our use of the open field arena developed by Simon and Dickinson (11) gave very different results to De et al's use of Petri dishes. The use of the latter for these kinds of observations is problematic because flies exhibit exploratory responses at the circumference of such chambers, rather than open field behavior, and in doing so

frequently occlude each other, confounding visual observations and automated tracking procedures (10). These concerns were addressed by Simon and Dickinson by re-designing an open field arena, which in our simulation studies clearly reveals a prominent A component. We conclude that De et al's assertion that the A component is an artifact is incorrect.

Indeed, even a superficial re-examination of the results presented by De et al (2013) reveal that they misinterpreted their own observations. We reproduce part of their Figure 2 in our Figure 5, placing their TriKinetics results below their corresponding visual monitoring of the same flies for easier comparison. While the two phenotypes displayed on the Y-axes are different, they should roughly correspond. From Figure 5, the visual observations suggest a peak of activity at around 09.00, 4 hours after the M peak in TriKinetics monitors. In addition the visually monitored 'M' and TriKinetics A components clearly overlap, so it seems astonishing that De et al misinterpreted this obvious result. De et al's own observations thus confirm that the A component is not an artefact.

We also further investigated the suggestion that the clock suppresses inappropriately high levels of activity under warm conditions, thereby generating higher amplitude A components in per^{01} mutants than the wild-type (4). We partially confirmed this observation in the extensive semi-natural data set of Vanin et al (3) for both per^{01} and tim^{01} when we regressed the amount of activity against temperature. We also observed much higher levels of the A component in Canton-S in warm temperature laboratory simulations than the wild isolate, HU, reflecting a similar observation in the natural dataset. Both A and E components show highly significant positive and negative correlations respectively with temperature in all strains. However, when we strictly isogenised each mutant background with one of our natural strains, HU, we did not observe a significant enhancement of A activity for per^{01}, but we did for tim^{01}. The *tim* gene encodes the light-sensitive negative regulator of the fly clock (21-23), but if this effect on summer activity is simply due to the flies lacking a clock, it is curious that per^{01} does not do the same. Consequently, it appears that the observation by Menegazzi et al that arrhythmic mutants may be unable to suppress the A component to the same extent as wild-type (4) may be generally correct, but this effect is significantly modulated by interactions with the genetic background and perhaps by the behavioral paradigm in which it is studied.

In addition, we studied the relative levels of M, A and E in flies carrying mutations in photo- and thermo-reception pathways. The levels of M, A and E are somewhat interdependent because each was taken as a proportion of total activity (including night-time) so as one component is elevated, another may be suppressed. Nevertheless, natural summer simulations revealed that *glass, trp* and *norpA* and the double mutant $glass^{60j}\ cry^{b}$ blunted the expression of M and E peaks and led to significant elevation of A (Fig S3). These observations resonate with Vanin et al's (3) results in semi-natural conditions in which the onset of the M component appeared to be a highly temperature-dependent response to the twilights with little clock input. As the absolute levels of A were significantly higher in mutants of *trp, norpA* and $glass^{60j},\ cry^{b}$ compared to the HU congenic controls, this suggests that their primary effect may be on A, so that under summer conditions, visual photoreceptor input suppresses the A component. Of the mutants that are known to be implicated in thermal sensing, *pyx* suppressed both M and E components but left A intact, whereas the most dramatic response was observed in $TrpA1^{1}$ mutants in which the A component was effectively eliminated. TrpA1 is a transient receptor potential channel previously implicated as an important nociceptor for both heat (24) and light (25). Lee & Montell described *TrpA1* expression within each subset of the canonical clock neurons (17) so we determined whether expression of *TrpA1* in clock cells was required to mediate the A peak. Down-regulation of *TrpA1* using the *timgal4* driver, enhanced by *UAS-Dicer2* had no effect on the amplitude of the A peak so it would appear that any expression of *TrpA1* in clock neurons is unlikely to contribute to the A component.

TrpA1 expression was initially found to be limited to a few brain cells, the sub-oesophageal ganglion and eight cells in the thoracic ganglion (26, 27). Two pairs of AC neurons expressing TrpA1 appear to be the main internal thermosensors but they also integrate temperature information from peripheral sensors (28). The AC sensors are activated by TrpA1 at $\sim$25°C but a second response is observed at 27°C which is generated by *pyrexia*-expressing neurons located in the second antennal segment and which synapse onto the AC neurons (28). Interestingly, when we used the *pyx* mutant in our behavioral assay, we found no effect on the A component, mirroring the observation that *pyx* is also not required in a temperature preference assay (28), but we did observe a significant suppression of M and E. *Painless* is also expressed in the antennae, but again we did not observe any effect on the A component in *pain* mutants. The rapid warmth response peripheral receptor Gr28b(d) which is located in the aristae (16) was also not required for the A component but, like *pyx,* suppressed M and E. We conclude that the peripheral sensors encoded by *pyx*, and *Gr28b(d)* may be involved in setting levels of M and E in circadian locomotor summer responses, but are not relevant to the A component. The circadian temperature entrainment mutant *nocte* is also largely irrelevant to the summer locomotor profile, but the effects of *norpA* which has similar temperature entrainment phenotypes to *nocte* are almost certainly due to its role in photoreception (15).

Modulation of the phase of the A component in *per* mutants has been observed by Vanin et al (3) and under some summer conditions by Menegazzi et al (4). One possible explanation is that in per^{s} (and per^{01}) mutants, the earlier A phase may simply represent a phase advance in the mutants for sensing the daily increase in temperature (4). As well as the four TrpA1 positive AC neurons that appear to act as internal thermosensors (26, 27), other TrpA1 positive cells also lie in dorsal regions in the vicinity of the DN clock cells (27). It remains to be seen whether any of the non-clock expressing TrpA1 neurons such as the AC or dorsal neurons have direct connections to the clock cells and if so, what the polarity of these interactions might be. It could be imagined that if clock cells send signals to the thermal sensors (or *vice versa*), then that might generate the phase changes that are observed in the A component in *per* mutants under natural conditions (3, 4).

In conclusion, the study of semi-natural circadian behavior in *D. melanogaster* initiated by Vanin et al (3) raised some interesting challenges to the canonical model of the clock developed under strictly artificial laboratory conditions. De et al's (8) suggestion that the A component is an artefact has been shown to be manifestly incorrect, both by our experiments, and by scrutiny of these authors' own results which they appear to have badly misinterpreted. Instead, we suggest that the molecular and physical basis of the A component appears to reside within the TrpA1 internal thermosensory neurons rather than those canonical clock neurons that may express TrpA1 nor the peripheral antennal temperature sensors. However all three locomotor components can be modulated by mutation in the photoreceptor and peripheral thermoreceptor pathways and the challenge will be to dissect the neuroanatomical pathways by which these sensors interact with clock cells (6, 7). In conclusion, the study of circadian behavior in semi-natural conditions in mammals (1, 2) and in flies (3, 4), as well as the modelling of natural circadian data (29) can inform and refine the current models of how clocks work at behavioral, ecological, anatomical and molecular levels.

Materials and Methods

Fly strains:

Flies were raised at 25°C in LD12:12 cycles. Congenic male per^{s}, per^{L} and per^{01} mutants were backcrossed for 6 generations to a w^{1118} that had itself been previously backcrossed for 10 generations to the wild Houten (HU) strain, isolated from the Netherlands in 2005 and maintained as isofemale lines (30). As *per* maps very close to *w,* we followed the *per* allele in each backcross generation by eye color and confirmed the final strains behaviorally in circadian locomotor assays. After 6 generations of backcrossing into HU, the residual genetic variation is 1/64 or less than 2%. All Gal4 lines had also been previously backcrossed to HU for 6 to 10 generations. Other mutant lines first had appropriate chromosomes replaced with those of HU using balancers, and then backcrossed to HU for two further generations before behavioral observations were made.

Outcrossed $glass^{60J}$ (31), cry^{b}, $norpA^{P41}$ (32) and $cry^{b,}$ $glass^{60J}$ double mutants from existing laboratory stocks, trp^{1} (stock #5692) (33), $painless^{EP2451}$ (stock #27895), $Gr28B^{MI}$ (stock #24190) and $TrpA1^{1}$ (stock #26504) were obtained from the Bloomington Drosophila Stock Center. $nocte^{P}$ and pyx^{3} were gifts from Ralf Stanewsky (UCL, London)

Behavioral observations:

Flies were anaesthetised with CO_2 and loaded into the experimental arenas. For TriKinetics and virtual beam breaking experiment, male flies were loaded in 10 cm long glass tubes, sealed with maize food and rubber bungs on one end and cotton wool at the other. Open field experiments used groups of four male and four female flies in 12 cm diameter circular arenas (11) with a central core of maize food (Fig S2). Activity arenas were placed into incubators and flies were allowed to recover and entrain to semi-natural conditions for at least 1.5 days before observations were made.

Natural light and temperature simulations:

We used a Memmert IPP500 peltier programmable incubator to smoothly cycle temperature and mimic a midsummer's day in northern Italy. We generated a reference temperature profile by taking the normalised average of three typical summer days in Treviso, Italy from Vanin et al (3) which could be baseline shifted to produce natural-like cycles of 20-30°C and 25-35°C (the correlation between the simulated and real data was >0.97, Fig S1). These profiles were converted to temperature cycles by the incubator's Celsius software. A light intensity regime to match that recorded in Treviso during midsummer (approximately LD 16:8) was generated using a custom-built programmable simulator (made by Stefano Bastianello, Euritmi, Venetian Institute of Molecular Medicine, Padova, Italy), with a peak intensity at 500 lux to resonate with light levels regularly recorded for summer observations in TriKinetics monitors placed in the shade (3). We used the Trikinetics environment sensor to record the light intensity but this would give slightly different maximum readings depending on exactly where the sensor was placed within the incubator, even though Trikinetics activity monitors were exposed to 500 lux. The spectral composition of the light matched that of natural midsummer light by combining outputs of 6 groups of LEDs with different emission spectra. Temperature was cycled to peak 2.5 h later than the light cycle peak, thereby mimicking natural midsummer recordings (3). Relative daily levels of the M, A and E locomotor components were calculated as in Menegazzi et al (4) to generate a measure of amplitude for each component by dividing up the day and taking the proportion of total daily activity (including night time activity) that fell into the corresponding daily segments.

Acquisition and analysis of video data:

Activity videos (1280x720 resolution at either 15 or 30 fps) were recorded under infrared light using a Logitech c930e webcam, modified to be sensitive only to light >850nm. To assess virtual beam crossings in unshaded conditions, 8, 10 cm glass tubes (same tubes as Trikinetics) were placed horizontally on a white background inside the incubator. The ActualTrack™ software divided the tube into two equidistant zones, and the number of fly movements from one zone into the other was tracked providing a measure of virtual beam crossings. Five minute long videos were recorded every 30 min across a two and a half day period. Data for all flies on both days were combined to calculate a daily group mean and SEM for each timepoint. For open field arena experiments, four male and four females were placed into the chamber (11) within the incubator, and 5 min long video time points were recorded every hour across a 24 h period. Videos were analysed with ActualTrack™ to record the total distance moved by each fly in that period. The mean fly activity at each time point was used to calculate a daily profile and an overall mean based on three replications was generated for each genotype. The ActualTrack™ settings used to track flies are described in more detail in the Supporting Information.

For TriKinetics experiments under simulated natural conditions, entrained flies were recorded over 5 days in DAM2 monitors. For each fly a median daily activity profile was generated using 30 min bins and the genotype mean and sem was calculated. When we re-interrogated our natural data from the Vanin et al study (3), as each day is different in terms of the environmental variation, we calculated the daily mean and sem activity (in 30 min bins) for each group of males. Statistical analysis was performed using Prism 6.05 (GraphPad Software Inc).

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